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Purification of crotonyl-CoA reductase from *Streptomyces collinus* and cloning, sequencing and expression of the corresponding gene in *Escherichia coli*Kimberlee K. WALLACE¹, Zhuo-Yao BAO¹, Hong DAI¹, Russell DIGATE², Gregory SCHULER², Marilyn K. SPEEDIE¹ and Kevin A. REYNOLDS¹¹ Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland at Baltimore MD, USA² Medical Biotechnology Center, Maryland Biotechnology Institute, University of Maryland at Baltimore MD, USA³ National Center for Biotechnology Information, National Institutes of Health, Bethesda MD, USA

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A crotonyl-CoA reductase (EC 1.3.1.38, acyl-CoA:NADP⁺ *trans*-2-oxidoreductase) catalyzing the conversion of crotonyl-CoA to butyryl-CoA has been purified and characterized from *Streptomyces collinus*. This enzyme, a dimer with subunits of identical mass (48 kDa), exhibits a $K_m = 18 \mu\text{M}$ for crotonyl-CoA and $15 \mu\text{M}$ for NADPH. The enzyme was unable to catalyze the reduction of any other enoyl-CoA thioesters or to utilize NADH as an electron donor. A highly effective inhibition by straight-chain fatty acids ($K_i = 9.5 \mu\text{M}$ for palmitoyl-CoA) compared with branched-chain fatty acids ($K_i > 400 \mu\text{M}$ for isopalmitoyl-CoA) was observed. All of these properties are consistent with a proposed role of the enzyme in providing butyryl-CoA as a starter unit for straight-chain fatty acid biosynthesis. The crotonyl-CoA reductase gene was cloned in *Escherichia coli*. This gene, with a proposed designation of *ccr*, is encoded in a 1344-bp open reading frame which predicts a primary translation product of 448 amino acids with a calculated molecular mass of 49.4 kDa. Several dispersed regions of highly significant sequence similarity were noted between the deduced amino acid sequence and various alcohol dehydrogenases and fatty acid synthases, including one region that contains a putative NADPH binding site. The *ccr* gene product was expressed in *E. coli* and the induced crotonyl-CoA reductase was purified tenfold and shown to have similar steady-state kinetics and electrophoretic mobility on sodium dodecyl sulfate/polyacrylamide to the native protein.

Keywords: crotonyl-CoA reductase; butyrate metabolism; *Streptomyces*; fatty acid biosynthesis.

Butyryl-CoA functions as an important building block in secondary metabolite formation. For example, *Streptomyces hygroscopicus* produces the immunosuppressant ascocin containing a butyrate-derived moiety, which distinguishes it from the related compounds FK506 and FK523 which contain a pantoate-derived and a propionate-derived unit, respectively, at the same structural position (Byrne et al., 1993; Tanaka et al., 1987; Hatanaka et al., 1988). Similarly, a butyrate-derived moiety differentiates the polyether antibiotic monensin A, produced by *Streptomyces cinnamonensis*, from monensin B which contains a propionate-derived moiety at the same structural position (Pospisil et al., 1983).

In secondary-metabolite-producing organisms, evidence for two pathways of butyryl-CoA formation have been presented (Fig. 1). One pathway involves isomerization of the valine catabolite isobutyryl-CoA to form butyryl-CoA. This pathway has been observed in the formation of monensin A (Pospisil et al., 1983; Reynolds et al., 1988), tylosin (Rezanka et al., 1988), jumomycin (Omura et al., 1983) and FK520 (Byrne et al., 1993),

suggesting that this isomerization may be general among streptomycetes. In monensin A biosynthesis, a series of *in vivo* and *in vitro* experiments demonstrated that this isomerization is likely a 1,2-vicinal rearrangement which is dependent on coenzyme B₁₂ (Reynolds et al., 1988; Brendelberger et al., 1988). However, there have been no reports of the purification and characterization of an enzyme catalyzing this rearrangement. The second pathway for butyryl-CoA formation appears to involve the condensation of two acetate units. The *in vivo* evidence for this pathway arises from the observation that [1-¹⁴C]acetate is incorporated into the butyrate-derived units of secondary metabolites, such as FK520 (Byrne et al., 1993), monensin A (Pospisil et al., 1983), and elaiophyllin (Gerlitz et al., 1992). The manner in which these butyrate units for secondary metabolism are derived from acetate is unknown although several possible pathways have been discussed (Gerlitz et al., 1992).

It has recently been shown that butyrate units (butyryl-CoA) are the likely starter units for straight-chain fatty acid biosynthesis in *Streptomyces collinus* and other *Streptomyces* (Wallace et al., 1995). A minor source for formation of this butyryl-CoA is from an isomerization of isobutyryl-CoA (Wallace et al., 1995). The pathway which provides the majority of the butyryl-CoA, however, has not been determined. On the basis of the secondary metabolic studies in streptomycetes it seems reasonable to suggest that this butyryl-CoA may originate from the condensation of two acetate units.

In mammalian mammary glands (Majra and Kumar, 1974) and *Euglena gracilis* (Inui et al., 1986), butyryl-CoA used for fatty acid biosynthesis is thought to be formed from the conden-

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Abbreviation. ORF, open reading frame.

Enzyme. Crotonyl-CoA reductase, acyl-CoA:NADP⁺ *trans*-2-oxidoreductase (EC 1.3.1.38).

Note. The novel nucleotide sequence data published here have been submitted to the GenBank and European Bioinformatics Institute (EB) sequence data bank and are available under the accession number U37135.

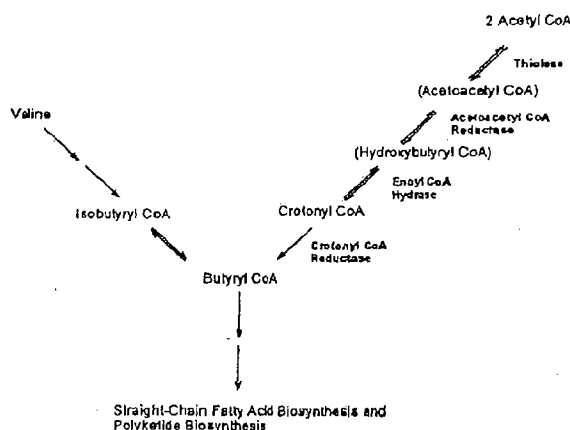


Fig. 1. Proposed role of a crotonyl-CoA reductase in butyrate metabolism in streptomycetes.

sation of two acetyl-CoA molecules. This pathway is apparently a reversal of the β -oxidation pathway for fatty acid degradation with one exception: the acyl-CoA oxidase (which converts butyryl-CoA to crotonyl-CoA) is replaced by an NADPH-requiring enoyl-CoA reductase which specifically converts crotonyl-CoA to butyryl-CoA (Podack and Seubert, 1974) (Fig. 1). This change favors the biosynthetic over the degradative pathway. A similar pathway may operate in streptomycetes. An active thiolase has previously been reported in cell-free extracts and $(\text{NH}_4)_2\text{SO}_4$ fractionations of *Streptomyces coelicolor* and an acetoacetyl-CoA reductase has previously been isolated, also from *S. coelicolor* (Packter and Flatman, 1983). A possible role of these enzymes in poly(hydroxybutyrate) synthesis has been suggested. However, it is also possible that these enzymes are also involved in butyryl-CoA degradation and biosynthesis. The key enzyme in this pathway of butyryl-CoA biosynthesis, crotonyl-CoA reductase, however, has not been reported for streptomycetes. We have located an enzyme in *S. collinus* which specifically catalyzes the conversion of crotonyl-CoA to butyryl-CoA. Here we report on its purification, characterization and regulation, together with the cloning, sequencing and expression of the corresponding gene in *Escherichia coli*.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and plasmids. *Escherichia coli* strain DH5 α and plasmid pUC18 were obtained from Life Technologies (Gaithersburg MD). *Streptomyces collinus* strain Tü 1892 was supplied by Professor H. Zährner (Tübingen) and Professor A. Zeeck (Göttingen). *E. coli* strain BL21(DE3), and plasmid pET3C were provided by Dr F. W. Studier (Brookhaven National Laboratory, New York).

Bacterial growth conditions for molecular cloning. *E. coli* was grown in liquid Luria-Bertani medium (37°C and 240 rpm) or on solid Luria-Bertani medium (1.5% agar) supplemented, when required, with ampicillin (100 $\mu\text{g}/\text{ml}$). Chromosomal DNA was isolated from *S. collinus* grown at 28°C in yeast extract malt extract medium (Hopwood et al., 1985).

DNA techniques. DNA manipulations were performed by standard procedures for both *Streptomyces* species (Hopwood et al., 1985) and *E. coli* (Sambrook et al., 1989). Oligonucleotides were designed based on known *Streptomyces* codon usage (Bibb

et al., 1984; Wright and Bibb, 1992) and were synthesized by the University of Maryland Biopolymer Laboratory. Contaminating salts in these preparations were removed using a Sephadex G-50 column. Oligonucleotides were labeled with ^{32}P using an end-labeling kit (Boehringer Mannheim) following the instructions obtained from the supplier.

DNA sequencing and analysis. Double-stranded DNA sequencing was performed by the dideoxynucleotide chain-termination method adapted for use with T7 DNA polymerase (Sequenase; Sanger et al., 1977; Zhang et al., 1988). Parallel incubations using dITP in place of dGTP were used to resolve ambiguities resulting from compressions. Computer-assisted analysis of the DNA sequences was performed using the programs of the Genetics Computer Group (Devereux et al., 1984). The BLAST family of programs (Altschul et al., 1990; Gish and States, 1993) was used to compare nucleotide and deduced amino acid sequences against the public sequence databases. Multiple sequence alignments were constructed from the MACAW program (Schuler et al., 1989). The probability (P) for each local alignment, providing the probability that the match is due simply to chance, was calculated according to statistical methods developed by Karlin and Altschul (1990, 1993).

Preparation of thioesters. Coenzyme A thioesters were prepared using the mixed anhydride method (Fong and Schultz, 1981). Formation of pantetheine thioesters was achieved in an analogous manner. The pantetheine was generated by overnight treatment of pantetheine (50 mg, 0.09 mmol) with sodium borohydride (4.5 mg, 0.118 mmol) in 2 ml water. The *N*-acetylcysteamine thioesters were prepared using cyclohexylcarbodiimide as previously described (Reynolds et al., 1992). Formation of the appropriate thioesters was confirmed by $^1\text{H-NMR}$ analysis.

Enzyme assays and protein concentrations. A portion of the protein solution (10–200 μl) was mixed with NADPH (10 ml of a 10 mM solution). The volume was adjusted to 1 ml with buffer A (50 mM potassium phosphate pH 7.5, 1 mM EDTA, 1 mM dithioerythritol and 10% glycerol), and the reaction mixture was equilibrated to 30°C. Crotonyl-CoA (0.1 mM), or other enoyl-CoA compounds, were added to initiate the reaction. Enzyme activity was measured spectrophotometrically by following the decrease in absorbance at 340 nm which corresponded to the oxidation of the nicotinamide cofactor. A unit (U) of enzyme activity is the amount of enzyme catalyzing the oxidation of 1 μmol NADPH/min. For kinetic studies, assays were conducted at least in duplicate for each data point. Saturating conditions of non-varied substrates were present in all assay mixtures. Protein concentrations were determined using the assay of Bradford (1976) with BSA as a standard.

Inhibition studies. Inhibition studies with divalent metal ions and thiol-blocking groups were conducted in a buffered solution of 50 mM Tris/HCl pH 6.5 and 10% (by vol.) glycerol. In these studies, the enzyme was incubated (5 min) with a 1 mM concentration of the metal prior to the addition of NADPH and substrate.

Inhibition by long-chain acyl-CoA compounds was conducted by incubating the enzyme (2 min) in the presence of the acyl-CoA inhibitor and NADPH. The reaction was initiated by the addition of crotonyl-CoA.

Determination of molecular mass of crotonyl-CoA reductase. The native molecular mass of the enzyme was determined using gel exclusion chromatography as previously described (Reynolds et al., 1992). The apparent subunit mass was determined by SDS/PAGE with a 12.5% polyacrylamide gel (Laemmli, 1970) with the following molecular mass markers (Sigma Chemical Company): bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase

(36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), trypsin inhibitor (20.1 kDa) and β -lactalbumin (14.2 kDa).

Growth of *S. collinus* for the harvest of crotonyl-CoA reductase activity. Seed cultures were prepared by transferring the spores from one slant to a 500-ml flask containing 100 ml medium A which contained 20 g soybean meal (full fat) and 20 g mannitol in 1 l tap water (pH 7.2). After incubation at 30°C and 280 rpm for 48 h, 40 ml of the seed culture was transferred to a 2-l flask containing 400 ml medium A. After 24 h, cultures were harvested for enzyme activity by centrifuging at 10000×g in a Beckman model J2-21 centrifuge. The supernatant was discarded and the cells were washed with buffer A. Cells were pelleted by centrifugation, and the washing step was repeated. The wet cell paste was frozen at -70°C until needed.

Purification of crotonyl-CoA reductase. *S. collinus* cells (600 g) were rapidly thawed in 320 ml buffer A containing 0.1 mM phenylmethylsulfonyl fluoride. The resulting cell suspension was passed through a French pressure cell (Aminco) at 80–100 MPa. This suspension was centrifuged at 30000×g and 4°C for 30 min. The supernatant was collected and the pellets of cellular debris were washed again with the same buffer. The combined supernatants afforded a cell-free extract (670 ml) which was loaded onto a DEAE-cellulose column (5.5×23 cm) that was subsequently washed with two column volumes of buffer A. The protein was eluted using a linear 1-l gradient of 0–1 M KCl. Active fractions containing crotonyl-CoA reductase activity eluted as a broad peak centered between 200–300 mM KCl. The active fractions were combined (110 ml), made up to 25% saturation with ammonium sulfate and stirred overnight with phenyl-Sepharose. The resin was washed with 400 ml buffer A containing 25% saturation of ammonium sulfate. Protein was eluted from the resin by successive washes (100–150 ml) of buffer A containing 20%, 15% and 10% ammonium sulfate saturation. The active fractions, which eluted in the 15% and 10% ammonium sulfate washes, were combined to yield 510 ml of protein solution. The volume was reduced to 37 ml by ultrafiltration through an Amicon PM10 membrane. This protein solution was divided into two aliquots and purified further using a Sephadex G-100 column (3×41 cm). Active fractions (83 ml) were combined, and guanidine was added to a concentration of 10 mM. The volume of the solution was reduced to 33 ml and loaded onto a DEAE-cellulose column (1.2×8.5 cm). The column was washed with 12 ml buffer A and developed using a 90-ml linear gradient of 0–450 mM KCl at a flow rate of 0.2 ml/min. The active fractions eluted as a peak centered around 270 mM KCl. These fractions (16 ml) were combined, diluted to 180 ml with Buffer A and reduced in volume to 31 ml by ultrafiltration through an Amicon PM10 membrane. Following volume reduction, guanidine was added to a concentration of 10 mM. The resulting solution was applied to a Pharmacia Mono Q HR 5/5 column. Following washing of the column with 15 ml buffer A, the protein was eluted with a 90-ml linear gradient of 0–450 mM KCl at a flow rate of 0.15 ml/min. The active fractions (2.8 ml), which eluted as a peak centered around 215 mM KCl were pooled and ammonium sulfate was added to 40% saturation. This protein solution was loaded onto a Pharmacia phenyl-Superose HR 5/5 column. The protein was eluted with a 90-ml linear gradient of 40–25% ammonium sulfate at a flow rate of 0.15 ml/min. The crotonyl-CoA reductase (2.7 ml) eluted in a narrow peak at 27% ammonium sulfate saturation.

Amino acid microsequencing. Purified crotonyl-CoA reductase was subjected to electrophoresis through a 12.5% polyacrylamide gel containing 0.1% SDS and transferred to a polyvinylidene difluoride (Immobilon) membrane (Matsudaira, 1987). The enzyme, located by staining with Coomassie blue, was ex-

cised and used for amino acid microsequence analysis (courtesy Dr W. Lane, Harvard University).

Cloning the *S. collinus* crotonyl-CoA reductase gene. A nondegenerate 60-base oligonucleotide probe (5'-ACCGTCAA-GGACATCCTCGACGCCATCCAGTCCAAGGACGCCACCT-CCGCCGACTTCGCC-3') that would likely encode the TVKD-ILDAIQSKDATSADFA peptide sequence of crotonyl-CoA reductase was designed and synthesized. This oligonucleotide probe was end-labeled to a specific activity of greater than 10⁴ cpm/ μ g and used in an overnight Southern hybridization (Southern, 1975) at 65°C with *S. collinus* DNA that had been digested with various restriction endonucleases. The hybridization buffer contained 0.9 M NaCl and 0.09 M sodium citrate. Post-hybridization wash procedures were performed according to standard protocols (Sambrook et al., 1989). A single 7-kb *Bam*HI fragment was identified. Fragments between 5–8 kb from a *Bam*HI digestion of *S. collinus* DNA were then collected from a 0.7% agarose gel by electroelution and cloned into pUC18. The resulting constructs were transformed into *E. coli* and a colony which harbored a plasmid pZYB2 (containing a 6.9-kb insert which hybridized to the ³²P-labeled nondegenerate 60-base oligonucleotide probe for crotonyl-CoA reductase) was detected by colony hybridization (Sambrook et al., 1989).

Construction of the crotonyl-CoA reductase overproducer *E. coli* BL21(DE3)/pZYB3. PCR was used to amplify the entire crotonyl-CoA reductase gene from pZYB2. One primer was a 25-base oligonucleotide containing a *Nde*I restriction site (underlined) and the first four codons of the crotonyl-CoA reductase gene, including the start codon (bold): 5'-CCG-GAGGCAACATATGACCGTGAAG-3'. The second primer was a 23-base oligonucleotide containing a *Bam*HI restriction site (underlined) and the complementary sequence corresponding to a region starting 26 bases downstream of the TGA stop codon of the gene: 5'-GCCACGGCCGGATCCTTCTGACG-3'. PCR amplification was performed using Vent DNA polymerase (New England Biolabs) and the buffer provided by the manufacturer in the presence of 200 mM deoxynucleoside triphosphate, 0.2 μ M of each primer, 1 μ g linearized pZYB2, and 2.5 U enzyme in a final volume of 100 μ l for 30 cycles with a thermal cycler. The first cycle consisted of 3 min of denaturation at 95°C, 2 min of annealing at 60°C, and 2 min of extension at 72°C. The second and subsequent 28 cycles had a denaturation step of 2 min and 1.5 min, respectively. The 1.4-kb PCR product was purified, digested with *Nde*I and *Bam*HI and ligated into pET3C to produce the plasmid pZYB3 (comprised of pET3C and the 1.4-kb *Nde*I–*Bam*HI *S. collinus* DNA fragment carrying the crotonyl-CoA reductase gene). This plasmid was used to transform *E. coli* BL21(DE3) and a single colony was selected for preparative production and subsequent purification of the enzyme.

Expression of crotonyl-CoA reductase by *E. coli* BL21(DE3)/pZYB3. *E. coli* BL21(DE3)/pZYB3 was grown at 37°C in Luria-Bertani medium containing ampicillin (100 μ g/ml). When the cells reached an *A*_{502 nm} of approximately 0.5 they were induced by addition of isopropyl β -D-thiogalactopyranoside to a final concentration of 0.5 mM and grown for a further 3 h (Studier et al., 1990).

Purification of the overexpressed crotonyl-CoA reductase. Cells obtained from a 3-h induction of two 1-l fermentations of *E. coli* BL21(DE3)/pZYB3 were pelleted by centrifugation at 6000 g for 10 min. The cells were lysed in buffer B (containing 50 mM Tris/HCl and 10%, mass/vol., sucrose at pH 8.0) with lysozyme (2 mg) in the presence of 0.1% Brij-58 for 30 min at 0°C. After centrifugation at 25000×g for 1 h the resulting cell-free extract (40 ml) was loaded onto a DEAE-cellulose column (5.5×15 cm) which had been equilibrated with

Table 1. Purification of crotonyl-CoA reductase from *Streptomyces collinus*. 1 unit (U) of enzyme activity is the amount catalyzing the oxidation of 1 μ mol NADPH/min.

Chromatographic step	Volume	Protein	Total activity	Specific activity	Purification
	ml	mg	mU	mU/mg	-fold
Crude extract	670	5034	4600	0.91	—
DEAE-cellulose	110	738	2100	2.8	3
Phenyl-Sepharose	510	200	3400	17	18
Sephadex G-100	83	60	2700	45	49
DEAE-cellulose	16	4.1	1100	268	282
Mono-Q	2.8	0.8	390	488	527
Phenyl-Superose	2.7	0.045	130	2889	3068

buffer A. The column was washed with buffer A (200 ml) and developed using a 600-ml linear gradient of 0–1 M KCl in buffer A. Crotonyl-CoA reductase activity eluted in a peak between 150–300 mM KCl. The fractions containing the highest activity were combined (38 ml) and divided into two 19-ml aliquots. Each aliquot (19 ml) was made up to 40% ammonium sulfate saturation over a 30-min period and loaded onto a Pharmacia phenyl-Superose HR 5/5 column. The column was washed with buffer A (10 ml) containing 40% ammonium sulfate saturation. Protein was eluted using a 90-ml linear gradient of 40–20% ammonium sulfate saturation in buffer A at a flow rate of 0.15 ml/min. The enzyme eluted in a peak centered around 28% ammonium sulfate saturation. All active fractions were combined (4 ml), diluted to 50 ml with buffer A and reduced in volume to 12 ml by ultrafiltration through an Amicon PM10 membrane. This protein solution was loaded onto a Pharmacia Mono Q HR 5/5 column and the column was washed with 10 ml buffer A. The purified crotonyl-CoA reductase activity eluted in the column wash.

RESULTS

Purification and preliminary characterization of crotonyl-CoA reductase from *S. collinus*. Crotonyl-CoA reductase activity was present in *S. collinus* cells harvested after 24 h of cultivation. The enzyme was purified to near homogeneity (Table 1) in six chromatographic steps. Accurate determination of enzyme activity in the early steps of the purification was hampered by a thioesterase that efficiently hydrolyzed the thioester bond of both crotonyl-CoA and butyryl-CoA. Enzyme activity was inhibited by ammonium sulfate; however, this inhibition was overcome by addition of 10 mM guanidine. The requirement for guanidine appeared specific for isolation of the enzyme from *S. collinus*, since ammonium sulfate inactivation was not observed when the enzyme was purified following overexpression in *E. coli*.

SDS/PAGE of the purified crotonyl-CoA reductase revealed a major band corresponding to a molecular mass of 48 kDa. Gel exclusion chromatography on a Superose 12 column demonstrated a molecular mass of 91 ± 8 kDa indicating that crotonyl-CoA reductase is most likely a homodimer. The crotonyl-CoA reductase was unable to convert butyryl-CoA to crotonyl-CoA in the presence of NADP under standard assay conditions. The enzyme was shown to be most active at 40°C (at this temperature the enzyme retained 47% of its activity after 30 min) and to have a pH optimum of 6.5.

Cofactor specificity. NADPH was the sole electron donor for the reduction catalyzed by crotonyl-CoA reductase. Incubation of the enzyme with NADH resulted in no observable activity.

The K_m value for NADPH was determined to be 15 ± 5 μ M, as calculated from three Eadie-Hofstee plots (Eadie, 1942; Hofstee, 1959) at crotonyl-CoA concentrations of 50, 100, and 150 μ M. Concentrations of NADPH above 200 μ M led to inhibition of enzyme activity. The enzyme activity was also inhibited by NADP, with a K_i of approximately 630 μ M, calculated from two double-reciprocal plots with NADP concentrations of 100 μ M and 150 μ M. No significant activation or inhibition of the activity of crotonyl-CoA reductase was observed upon addition of either flavin adenine dinucleotide (18 and 72 μ M) or flavin mononucleotide (13 and 130 μ M). This observation and the lack of any spectrophotometric properties in the visible range suggest that the enzyme does not require a flavin prosthetic group.

Substrate specificity. The enzyme exhibited a high substrate specificity for crotonyl-CoA, a short-chain-length (C_4) enoyl-CoA compound. No measurable activity was observed with the shorter-chain-length (C_3) enoyl-CoA thioester, acryloyl-CoA. Similarly, medium- or longer-chain-length enoyl-CoA thioesters (*trans*-2-pentenoyl-CoA, *trans*-hexenoyl-CoA, *trans*-2-octenoyl-CoA, *trans*-2-dodecenoyl-CoA, *trans*-2-hexadecenoyl-CoA), at concentrations of 200 μ M, did not react measurably with the enzyme (rate observed was less than 1% of that observed using crotonyl-CoA as the substrate). The enzyme was unable to reduce either the *N*-acetylcysteine or the pantetheine thioester of crotonic acid. The K_m value determined for crotonyl-CoA was 18 ± 4 μ M as calculated from three Eadie-Hofstee plots (Eadie, 1942; Hofstee, 1959) at NADPH concentrations of 23 μ M, 46 μ M, 184 μ M.

Inhibition by divalent cations and thiol group reagents. A 5-min incubation of the enzyme with various divalent cations (1 mM), prior to addition of substrate and NADPH, led to significant inhibition (MgCl₂, 30% inhibition; CoCl₂, 100%; ZnCl₂, 55%; MnCl₂, 100%; and CaCl₂, 100%). These cations were considerably less effective as inhibitors if they were added at the same time as the NADPH and substrate.

A 30-min incubation of crotonyl-CoA reductase with the thiol group inhibitor β -chloromercuribenzoate at a concentration of 8 μ M led to approximately 85% inhibition of enzyme activity. Addition of either crotonyl-CoA or NADPH to this incubation afforded virtually complete inhibition protection. Iodoacetamide and *N*-ethylmaleimide (1 mM) were also shown to be inhibitory to crotonyl-CoA reductase activity (40% and 80% inhibition respectively).

Inhibition by CoA thioesters of straight-chain fatty acids. As shown in Table 2, incubation of the enzyme in the presence of CoA thioesters containing 12–20 carbon atoms resulted in inhibition of enzymic activity. The greatest degree of inhibition was observed in the presence of palmitoyl-CoA and myristoyl-CoA.

A		
CCR	mtvkdiidaigskdatsadfaalqlpesyraitvkhkdetemfagletredkprksihIDEVPELPGELALVAVMASSV	80
ADH3-Sce	-----mlrtstlftkrvqpslfsxnilrlqstasipktqkvifyenqnlhyKDIPVPEPKPNEILINVKYSGV	70
ADH-Kma	-----mlrtstlftkrvqpslfsxnilrlqstasipktqkvifyenqnlhyKDIPVPEPKPNEILINVKYSGV	69
QOR-Hsa	-----mlrtstlftkrvqpslfsxnilrlqstasipktqkvifyenqnlhyKDIPVPEPKPNEILINVKYSGV	47
QOR-Eco	-----mlrtstlftkrvqpslfsxnilrlqstasipktqkvifyenqnlhyKDIPVPEPKPNEILINVKYSGV	38
B		
CCR	nynsvvtsifepvstfaflerygklspltkrhdlpyhiIGSDLAGVVLRTGPGVNAWQPGDevvahclsvelespdghdd	160
ADH3-Sce	chtdlhawhgdwplvpklplv-----GGHEGAGVVVKLGSNKGWKGdlagikwingscmtcefcas	133
ADH-Kma	chtdlhawhgdwplvpklplv-----GGHEGAGVVVKLGSNKGWKGdlagikwingscmtcefcas	132
QOR-Hsa	npvetyikagtyarkplpyt-----PGSDVAGVIEAVGDNASAFKKGdrvfctas	97
QOR-Eco	glnfidtyirsglypppslpsg-----LGTEANGIVSKVSGVKKHKAQdrvvyaq	89
C		
CCR	tmdpegrwgf-TNFGGLAETALVKTNLMPKRLITWEEAAGPLVNSTAYRQLvsrNGAAMKQGDVNLTWGASGGL	239
ADH3-Sce	ghesncpdadlsgyTHDGSFQQFATADAIQAQKIQQGTDLAEVAPILCAGVTYKAL---KEADLKAGDWVAISGAAGGL	210
ADH-Kma	sneancpdadlsgyTHDGSFQQYRTADAVQAARIEFGTDLAEVAPILCAGVTYKAL---KSANLKAGDWVAISGAAGGL	209
QOR-Hsa	-----TISGGYAEYALADHTVYKLEKDFKQGAAGIPIFYTAYRALI---HSACVKAESVYLVHGASGGV	161
QOR-Eco	-----SALGAYSSVHNIADKAAIIFAAISFEQAAASFLKGLTYVYLL---KTYEIKPDEQFLTHAAAGGV	153
D		
CCR	GSYATQFALAGGANPICVVSFPQAEICRSHGAEAIIDRnaegykwkdehtqdpkewkrfgKRIRLETGGEDTDIVFEH	319
ADH3-Sce	GSIAVQYATANGYRVVGLIDAGEEERLFFKLGGVFDFTktdkmv-----SDIQEATKGGPHGVINVS	274
ADH-Kma	GSIAVQYATANGYRVVGLIDAGEEERLFFKLGGVFDFTktdkmv-----SDIQEATKGGPHGVINVS	273
QOR-Hsa	GLAACQIARAYGLKILGTACTEEOQITVLQNGAHEVENHrevnyl-----DKIKKYVGEKGIDIIITEM	224
QOR-Eco	GLIACQWAKALGAKLIGTVGTAKQAQSAIKAGAWQVINYreedlv-----ERLKEITGGKKRVVYDS	216
E		
CCR	PGRETFGASVYVTRKGGTITTCAsstsgmheydnrylwnslkrligshfanyreayeanrliaqkhiptlsktyseet	399
ADH3-Sce	VSEAAISLSTETVVRPGCTVVLVGLpanayvksevfshvksinikgsyvgnradtrealdffsrglikspikivglseip	354
ADH-Kma	VSEAAINASTQFVRPTGTIVVLVGLpagaviksevfshvksinikgsyvgnradtreainffanghvhspikvvglseip	353
QOR-Hsa	LNVNLSKDLSSLHSGORVIVVSGrtgieinprdtakessligvtlfsstkeefqyaaalqagmeigwlpkvigsqyp	304
QOR-Eco	VGRDTWERSLDCLQRRLMVSPGNSsgavtgvnlgilnqkgslyvtrpslqgyittreelteasneifsliaagvikvdv	296

Fig. 2. Multiple alignment of the crotonyl-CoA reductase with three members of the quinone oxidoreductase superfamily. Multiple sequence alignments were performed with the MACAW program (Schuler et al., 1989). Abbreviations used are as follows: ADH3, alcohol dehydrogenase 3 from *Saccharomyces cerevisiae* (Young and Pilgrim, 1985); ADH-Kma, alcohol dehydrogenase of the yeast *Kluyveromyces fragilis* (Salicrú et al., 1991); QOR-Hsa, human ζ -crystallin quinone reductase (Gonzalez et al., 1993); QOR-Eco, quinone oxidoreductase of *E. coli* (Blatner et al., 1993). Amino acids within the seven blocks of related sequences are indicated in capital letters. Residues identical in four or more of the sequences are indicated in bold face. Dashes indicate gaps introduced to maximize alignment. The GxGxxAxxxNADP(H) binding motif of some dehydrogenase is located in block D (Scrutton et al., 1990; Wierenga et al., 1986).

Table 2. Inhibition of crotonyl-CoA reductase activity by straight-chain and branched-chain fatty acid thioesters. Experiments were conducted with 200 μ M crotonyl-CoA and 100 μ M fatty acid thioester, with the exception of the isopalmitoyl-CoA and isomyristoyl-CoA experiments (125 μ M crotonyl-CoA and 50 μ M fatty acid thioester). Long-chain acyl-CoA compounds were incubated with the enzyme for 2 min prior to addition of crotonyl-CoA. Enzyme activity was assayed spectrophotometrically by monitoring the decrease in absorbance at 340 nm.

Fatty acid thioester	Activity relative to control
Control	1
Lauroyl-CoA (12:0)	0.75
Myristoyl-CoA (14:0)	0.36
Palmitoyl-CoA (16:0)	0.24
Stearoyl-CoA (18:0)	0.92
Arachidoyl-CoA (20:0)	0.86
Isomyristoyl-CoA (14:0)	0.78
Isopalmitoyl-CoA (16:0)	0.95

the major straight-chain fatty acids produced by *S. collinus*. The branched-chain fatty acids, isopalmitoyl-CoA and isomyristoyl-CoA at a concentration of 50 μ M, were less effective inhibitors of the crotonyl-CoA reductase. Palmitoyl-CoA inhibited crotonyl-CoA reductase with a K_i of $9.5 \pm 6 \mu$ M (as calculated from

double-reciprocal plots at palmitoyl-CoA concentrations of 20 μ M and 35 μ M). The K_i value determined for myristoyl-CoA was $17 \pm 5 \mu$ M (using myristoyl-CoA concentrations of 35 μ M and 50 μ M). In contrast, K_i values in the range of 0.5 mM were determined for isopalmitoyl-CoA and isomyristoyl-CoA using the overexpressed protein (described below). Crotonyl-CoA reductase activity was slightly inhibited by the reaction product butyryl-CoA with a K_i of 0.9 mM (as calculated from a double-reciprocal plot with a butyryl-CoA concentration of 400 μ M).

Peptide sequence. The following high-confidence N-terminal sequence was obtained for crotonyl-CoA reductase: Thr-Val-Lys-Asp-Ile-Leu-Asp-Ala-Ile-Gln-Ser-Lys-Asp-Ala-Thr-Ser-Ala-Asp-Phe-Ala.

Molecular cloning and sequencing of the crotonyl-CoA reductase gene. A nondegenerate 60-base oligonucleotide probe was designed and synthesized based on the N-terminal peptide sequence of crotonyl-CoA reductase. This oligonucleotide probe was labeled with 32 P and used to identify a single 6.9-kb *Bam*HI fragment of *S. collinus* genomic DNA. A partial genomic library was made by ligating size fractionated *Bam*HI-digested *S. collinus* DNA (5–8 kb) into pUC18 and using the constructs to transform *E. coli* DH5 α . Colony hybridization and screening with the 60-base oligonucleotide probe led to the isolation of a single clone harboring a plasmid (pZYB2) with a 6.9-kb insert which

Table 3. Purification of the recombinant crotonyl-CoA reductase. 1 unit (U) of enzyme activity is defined as the amount catalyzing the oxidation of 1 μ mol NADPH/min.

Chromatographic step	Volume	Protein	Total activity	Specific activity	Purification
	ml	mg	mU	mU/mg	-fold
Crude extract	40	90	30 000	333	
DEAE-cellulose	19	50	26 300	526	1.6
Phenyl-Superose	4	24	18 800	783	2.3
Mono Q	19	3.8	12 600	3316	10.0

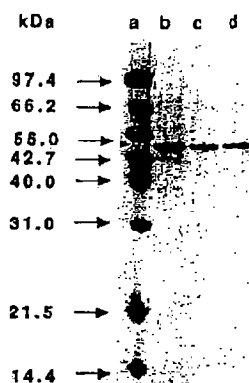


Fig. 3. SDS/PAGE of the purification of the recombinant crotonyl-CoA reductase from *E. coli* BL21(DE3). Lanes: (a) protein molecular mass standards [phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), glutamate dehydrogenase (55.0 kDa), ovalbumin (42.7 kDa), aldolase (40.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa)]; (b) DEAE-cellulose pool; (c) phenyl-Superose pool; (d) Mono-Q pool.

hybridized strongly to the probe DNA in Southern blots. A 1.5-kb downstream region, and a 200-base upstream region, adjacent to the sequence which hybridized to the 60-base oligonucleotide probe was sequenced.

Analysis of this sequence information revealed an ORF (open reading frame) of 1344 bp, corresponding to 448 amino acids and a predicted molecular mass of 49.4 kDa. Analysis of the DNA sequence within this ORF using the CodonPreference program demonstrated that it conformed to the established patterns of G+C bias and preferred codon usage for open reading frames in *Streptomyces* (Wright and Bibb, 1992; Bibb et al., 1984). The deduced amino acid sequence includes the previously determined N-terminal peptide sequence obtained for crotonyl-CoA. The ORF initiates with a GTG start codon and is located seven nucleotides downstream of a putative *Streptomyces* ribosomal binding site with the sequence GGAGG, complementary to the sequence at the 3'-terminus of the 16S rRNA of *S. lividans* (Bibb and Cohen, 1982; Hopwood et al., 1986).

Sequence analysis of crotonyl-CoA reductase. Comparison of the deduced amino acid sequence of crotonyl-CoA reductase against the protein database revealed statistically significant ($P < 10^{-6}$) similarities with members of the quinone oxidoreductase superfamily, also known as lens crystallins in certain mammals. Examples of this class of sequences bearing significant similarity to crotonyl-CoA reductase include *E. coli* quinone oxidoreductase (Blatner et al., 1993) ($P = 1.5 \times 10^{-13}$), alcohol dehydrogenases of the yeast *Kluyveromyces fragilis* (Saliola et al.,

1991) ($P = 1.9 \times 10^{-14}$), and human ζ -crystallin (Gonzalez et al., 1993) ($P = 2.2 \times 10^{-14}$). A multiple alignment of these sequences shows five dispersed blocks of high similarity (labeled A–E in Fig. 2) rather than a global relationship. Block A has the lowest significance ($P = 3.6 \times 10^{-15}$), but the significance of the remaining four blocks is extremely high ($P = 10^{-30}$). The highly conserved NAD(P)H consensus binding motif of GXGXXAXXXA (Hanukoglu and Gutfinger, 1989; Scrutton et al., 1990; Wierenga et al., 1986) was found in block D. The deduced amino acid sequence of the *ccr* product showed the strongest similarity, localized in these same short blocks, to the products of two putative open reading frames, ORF2 ($P = 2.1 \times 10^{-29}$) and ORF4 ($P = 2.9 \times 10^{-19}$), of the *actV1* gene cluster of *Streptomyces coelicolor* (Fernández-Moreno et al., 1994). Significant, but less striking, similarity of crotonyl-CoA reductase with enoyl-thioester reductases involved in 6-deoxyerythronolide B ($P = 6.2 \times 10^{-7}$) (Donadio et al., 1993) and fatty acid synthesis ($P = 4.0 \times 10^{-6}$) (Chang and Hammes, 1989; Holzer et al., 1989; Yuan et al., 1988) was also observed, centered around the NAD(P)H consensus binding motif.

Expression of the crotonyl-CoA reductase in *E. coli*. Confirmation of the identity of the isolated gene was obtained by expression of the putative crotonyl-CoA reductase in *E. coli*. PCR was used to amplify the entire crotonyl-CoA reductase gene and to introduce concomitantly an *NdeI* restriction site at the ATG start codon and a *BamHI* site downstream from the TGA stop codon. The PCR product of the predicted size (1.4 kb) was ligated into a transient expression vector pET3C, such that it was under the control of a bacteriophage T7 RNA polymerase promoter (Studier et al., 1990). The resulting construct, pZYB3 was used to transform *E. coli* BL21(DE3). SDS/PAGE analysis of a cell-free extract generated from the resulting *E. coli* BL21(DE3)/pZYB3 taken 3 h after induction with 0.5 mM isopropyl thiogalactoside revealed an intense band for a 48.0-kDa protein which was absent from extracts of a control culture of *E. coli* BL21(DE3). This crude cell-free extract of *E. coli* BL21(DE3)/pZYB3 was shown to possess an approximate specific activity of crotonyl-CoA reductase of 333 mU/mg protein (Table 3). Cell-free extracts of *S. collinus*, by contrast, exhibit a specific activity of crotonyl-CoA reductase of only 0.91 mU/mg protein. No measurable activity could be obtained with cell-free extracts of *E. coli* BL21(DE3) not carrying pZYB3.

Purification and preliminary characterization of the recombinant crotonyl-CoA reductase. A cell-free extract was generated from a 1-l fermentation of *E. coli* BL21(DE3)/pZYB3. 3 h after induction. The recombinant crotonyl-CoA reductase was subsequently purified approximately tenfold in three chromatographic steps (Table 2) to a final specific activity of 3316 mU/mg. An SDS/PAGE analysis of the purified enzyme revealed a single major band with a predicted molecular mass of 48.0 kDa (Fig. 3). The native protein size determined by analysis of the protein on Superose 12 was 85.0 ± 10.0 kDa, indicating a homo-

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